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Characterization of the Murine Antibody Response to Peptides Representing the Variable Domains of the Major Outer Membrane Protein of *Chlamydia pneumoniae*

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In an attempt to gain more knowledge about the immunogenicity of the variable domains (VDs) of the major outer membrane protein (MOMP) of *Chlamydia pneumoniae*, peptides representing these areas were used to immunize BALB/c and C57BL/6 mice. Antisera to the peptides and to peptides conjugated to keyhole limpet hemocyanin (KLH) were characterized by their ability to recognize the immunizing peptide and elementary bodies (EBs) of *C. pneumoniae* by enzyme-linked immunosorbent assay (ELISA) and Western blot (immunoblot). In addition, antiserum was analyzed for its molecular specificity by a pepscan as well as its in vitro neutralizing ability. In general, results obtained with antisera to the peptides paralleled the results obtained with the antisera to the KLH-conjugated peptides except that the titers or strength of reaction in the assays was less. Antisera to the VDs in both strains of mice gave ELISA titers to the homologous VD peptide ranging from 1,000 to >64,000. The strength of reactivity with the reduced MOMP as judged by Western blot, in most cases, paralleled the ELISA titer to the peptide. However, only antisera raised in both strains of mice to the VD1 and VD4 peptides reacted strongly with the EBs, suggesting surface exposure of these VDs. In addition, antisera to VD3 from C57BL/6 mice gave strong reactivity to EBs. By pepscan analysis antisera from both strains of mice reacted with several VD1 and VD3 octameric peptides, with weaker reactivity being seen with the octameric peptides in the other two VDs. This was in contrast to antisera raised to EBs of *C. pneumoniae* TW-183, which identified two immunogenic regions, one in VD1 and the other mapped to VD4. While antisera raised to EBs strongly neutralized the infectivity of *C. pneumoniae*, none of the peptide antisera was able to neutralize. In addition, peptides to the VDs were not able to block the neutralizing ability of the antisera to EBs of *C. pneumoniae*. Therefore, these results suggest that the VDs of the MOMP of *C. pneumoniae* are surface exposed but do not elicit neutralizing antibodies when linear peptides representing them are used as the immunogen.

The genus *Chlamydia* is composed of four species, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, and *Chlamydia pecorum*. While distinct differences exist among the species, members of the genus share many similarities including the characteristic growth cycle within host cells and common antigens (3, 10, 11, 16). The best-studied of the species is *C. trachomatis* because of its importance as a human pathogen in both ocular and genital infections. The recently described *C. pneumoniae* is also a human pathogen that causes respiratory infections and has been associated with coronary artery disease (8, 12).

In *C. trachomatis* and *C. pneumoniae* the major outer membrane protein (MOMP) is the most abundant of the membrane proteins (1, 15). Because of its potential importance in host cell interactions, this protein has been extensively studied in *C. trachomatis* and has been considered as a vaccine candidate (17, 21). DNA sequencing of the MOMPs from the different serovars of *C. trachomatis* has shown there to be four variable domains (VDs) interspersed with conserved or constant domains (24). The DNA sequence of the MOMP of *C. pneumoniae* can be aligned with the constant domains and VDs of the MOMP from *C. trachomatis*. However, on the basis of the uniform VD4 sequence among isolates of *C. pneumoniae* tested so far, it appears that variation within the VDs is limited in this species (5).

Monoclonal antibodies (MAbs) recognizing MOMP and polyclonal sera raised to peptides representing the VDs of MOMP of *C. trachomatis* have been shown to neutralize the infectivity of this organism both in vivo and in vitro (17, 21, 27). To date, no antibodies which can recognize the MOMP of *C. pneumoniae* and neutralize its infectivity have been described. In this report synthetic peptides representing the *C. pneumoniae* MOMP VDs were assessed for their immunogenicity and ability to elicit neutralizing antibodies.

MATERIALS AND METHODS

Organisms. *C. pneumoniae* TW-183 was obtained from the University of Washington Foundation, grown in HEp-2 229 cells, and frozen at −70°C in sucrose-phosphate-glutamate (pH 7.4) as previously described (19).

Peptides. Synthetic peptides, representing the four VDs of the MOMP of *C. pneumoniae*, were obtained from the University of California Microchemical Core Laboratory (14). Peptides were made with 9-fluorenylmethoxycarbonyl amino acids by using FASTMoc chemistry at a 0.1 mM scale on an Applied Biosystems (Foster City, Calif.) 430A automated peptide synthesizer. Resulting peptides were purified by high-pressure liquid chromatography with a Vydac C_{18} column using 0.1% trifluoroacetic acid in water and 0.1% acetonitrile. All peptides had an N-terminal cysteine added to the native sequence. The amino acid sequences of the peptides used are as follows: VD1, C-DAPKTFSMGAKPT GSAAANYTTAVDRPNPA; VD2, C-GLFGVKGTTVNANELPNVSLSNG VVEL; VD3, C-GYKGVAFPLPTDAGVATATGTK; VD4, C-QPKLPTAVL NLTAWNPSLLGNATALSTTDSFSDFM. In some experiments the peptides were conjugated to keyhole limpet hemocyanin (KLH) by using the Imject maleimide-activated immunogen conjugation kit (Pierce, Rockford, Ill.). The peptides were stored dry at −20°C until reconstituted in sterile phosphate-

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TABLE 1. Titers of antisera to VD MOMP peptides on day 56[a]

Peptide	ELISA titer (10^3)				Western blot[b]	
	BALB/c		C57BL/6			
	Homologous peptide[c]	EBs of TW-183[d]	Homologous peptide[c]	EBs of TW-183[d]	BALB/c	C57BL/6
VD1	0	0	4	0.1	0	4+
KLH-VD1	32	1.6	>64	12.8	4+	4+
VD2	8	0	64	0	2+	2+
KLH-VD2	16	0	32	0.2	2+	2+
VD3	2	0	1	0	1+	3+
KLH-VD3	16	0	16	3.2	4+	4+
VD4	4	1.6	4	0.4	3+	3+
KLH-VD4	2	1.6	8	0.8	3+	3+

[a] Pooled antisera from four mice were used for each peptide or KLH-peptide.
[b] Intensity was graded from 1+ to 4+ of reaction with a 1:200 dilution of antiserum.
[c] Lowest dilution tested, 1:500.
[d] Lowest dilution tested, 1:100.

buffered saline (PBS) (0.01 M, pH 7.4), frozen at −70°C, and used within 3 months.

Immunization of mice. Six-week-old BALB/c (H-2^d) mice (Simmonsen, Gilroy, Calif.) or C57BL/6 (H-2^b) mice (Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally with either 10^7 inclusion forming units (IFU) of *C. pneumoniae* TW-183 or 100 μg of peptide or KLH-conjugated peptide suspended in complete Freund's adjuvant (Sigma). Each group consisted of four or five mice. Animals were boosted intraperitoneally on days 14, 28, and 42 with 100 μg of peptide or KLH-peptide in incomplete Freund's adjuvant (Sigma) or on days 14 and 28 with 10^7 IFU of *C. pneumoniae*. Control mice were injected with KLH by using the same protocol as described for the mice immunized with the peptide. Animals immunized with the peptides were bled on days 7, 14, 28, 42, 56, and 67. Animals immunized with *C. pneumoniae* were bled on days 14, 21, 35, and 42. Serum was separated and frozen at −70°C.

Immunoassays. The indirect immunofluorescence assay (IFA) was performed as previously described (18) with the following modifications. Briefly, *C. pneumoniae* (TW-183) was used to inoculate HEp-2 cells so that 50% of the monolayer was infected. After 48 h of incubation the monolayers were removed from the flask with trypsin; washed in Eagle's minimal essential medium containing Earle's salts; resuspended in the same medium supplemented with 10% fetal bovine serum, glutamine, and gentamicin (50 μg/ml); and applied to 5-mm wells on a glass slide. Slides were incubated in a humid chamber at 37°C for 8 h or until a confluent monolayer coated the slide well surface. Slides were then washed with PBS and fixed in acetone for 10 min. Slides were stored dry at −70°C. For performance of the IFA, slides were allowed to reach room temperature before the addition of the antisera. Primary and secondary antibody incubations were at 37°C for 30 min and were followed by several washes in PBS. Control slides were stained with a fluorescence-tagged mouse MAb directed to chlamydial lipopolysaccharide (LPS) (Ortho Diagnostics Systems, Inc., Raritan, N.J.).

Western blots were performed as previously described (19). Antigens were prepared by harvesting 72-h *C. pneumoniae*-infected HEp-2 cells contained in 175-cm^2 flasks. Antigens were sonicated in sucrose-phosphate-glutamate for three 20-s intervals followed by low-speed centrifugation at 700 × *g* for 10 min. The resulting supernatant was layered over a 35% Renografin column, the resulting pellet was washed with PBS, a protein determination was performed, and the antigen was stored at −70°C until needed. Elementary bodies (EBs) were resolved by 10% Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (23). Electrophoretic transfers were made with Trans-Blot SD (Bio-Rad Laboratories, Richmond, Calif.).

Overlapping peptides representing the VDs were synthesized by the method of Geysen et al. (6, 7) using the recommendations supplied in the commercially available epitope mapping kit (Cambridge Research Biochemicals, Cambridge, England). Enzyme-linked immunosorbent assays (ELISAs) were performed with the pins containing the overlapping octameric peptides by using mouse polyclonal sera at a dilution of 1/500 as the source of the primary antibodies as previously described (17). Upon incubation and subsequent washing, peptide pins were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Organon Teknika, Philadelphia, Pa.). Pins were then washed, and the optical density was read at 405 nm with an ELISA reader (Bio-Rad Laboratories) using 2,2'azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as the substrate. After assays in which strong antibody binding was detected on the pins the efficiency of the antibody removal step using SDS, 2-mercaptoethanol, and sonication was monitored by performing an ELISA with only the second antibody. If reactivity was still detected, the pins were retreated until the signal returned to background levels.

In vitro neutralization assays. The neutralization assays used have been previously described (17, 19). All reactions, except as noted, were performed in a PBS diluent containing 5% guinea pig serum as a source of complement. *C. pneumoniae* was allowed to incubate at 37°C for 45 min with dilutions of the antisera. Subsequently, these mixtures were used to inoculate monolayers of HeLa 229 or HEp-2 cells on coverslips contained in 1-dram (15 by 45 mm) glass vials which had been rinsed with 1 ml of PBS. Inhibition studies were performed by incorporating 2 μg of peptide per ml into the initial preincubation of the organism with the antiserum (17). Monolayers were centrifuged at 2,000 × *g* for 1 h and incubated at 37°C for 1 h, and then Eagle's minimal essential medium containing Earle's salts, fetal bovine serum, glutamine, gentamicin, and cycloheximide (1 μg/ml) was added. Infected cultures were incubated for 48 h, fixed with methanol, and stained by an indirect method using a mixture of two MAbs, CP-33 and CP-20, raised in our laboratory that recognize the LPS and a 33-kDa protein of *C. pneumoniae*, respectively, and an anti-mouse horseradish peroxidase-conjugated antibody. In most cases, IFU at each dilution in the anti-KLH-treated group were taken as the control for the corresponding IFU counts obtained at each dilution of the anti-peptide or anti-KLH-peptide group.

RESULTS

Overall results. The peptides corresponding to the four VDs were immunogenic in both BALB/c mice and C57BL/6 mice. The overall results for peptides alone and peptides conjugated to KLH can be found in Table 1. In general, the VD peptides compared with their KLH-conjugated equivalents gave weaker responses as judged by ELISA using the peptide as the antigen. While there was some variation in titers to the peptides, with the C57BL/6 mice giving stronger responses, the relative results with the two mouse strains were similar. However, when EBs were used as the antigen in an ELISA, while both the anti-KLH-VD1, anti-VD4, and anti-KLH-VD4 sera from the two strains reacted with the EBs, only anti-KLH-VD2 and anti-KLH-VD3 sera from C57BL/6 mice were able to recognize the chlamydial organisms, although the KLH-VD2 antiserum reaction was weak. As would be expected, in general, when antiserum was used to probe EBs in a Western blot in which conformational epitopes were minimized, the strength of reactivity to the MOMP as seen in Fig. 1 paralled that of the ELISA titers to the short peptides but not the titers to the EBs.

VD1. The VD1 peptide was weakly immunogenic in C57BL/6 mice and nonimmunogenic in BALB/c mice. However, the KLH-VD1 peptide was the most immunogenic of the VD peptides in both strains of mice as seen in the titers to the peptide and the strength of reaction to MOMP in Western blots. In addition, this antisera reacted with the EBs in an ELISA and therefore recognized epitopes on the EBs that are surface exposed. Supporting this finding, by inclusion IFA the KLH-VD1 antiserum from C57BL/6 mice was also positive with a titer of 320. A pepscan of the KLH-VD1 antisera from both strains of mice revealed two main areas of recognition, the greatest being that for the peptide TGSAAANYTT (Fig.

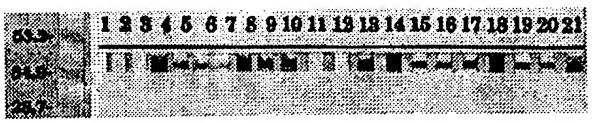

FIG. 1. Western blot of *C. pneumoniae* EBs probed with pooled antisera from four or five mice to the VD peptides or EBs. Antisera from BALB/c mice are in lanes 1 to 10 and 21. Antisera from C57BL/6 mice are in lanes 11 to 20. Control sera raised to Freund's complete and incomplete adjuvants are in lanes 1 and 11, respectively, and control sera to KLH suspended in Freund's adjuvant are in lanes 2 and 12. Antisera to the different antigens are in the following lanes: VD1, lanes 3 and 13; KLH-VD1, lanes 4 and 14; VD2, lanes 5 and 15; KLH-VD2, lanes 6 and 16; VD3, lanes 7 and 17; KLH-VD3, lanes 8 and 18; VD4, lanes 9 and 19; KLH-VD4, lanes 10 and 20; *C. pneumoniae* EBs, lane 21.

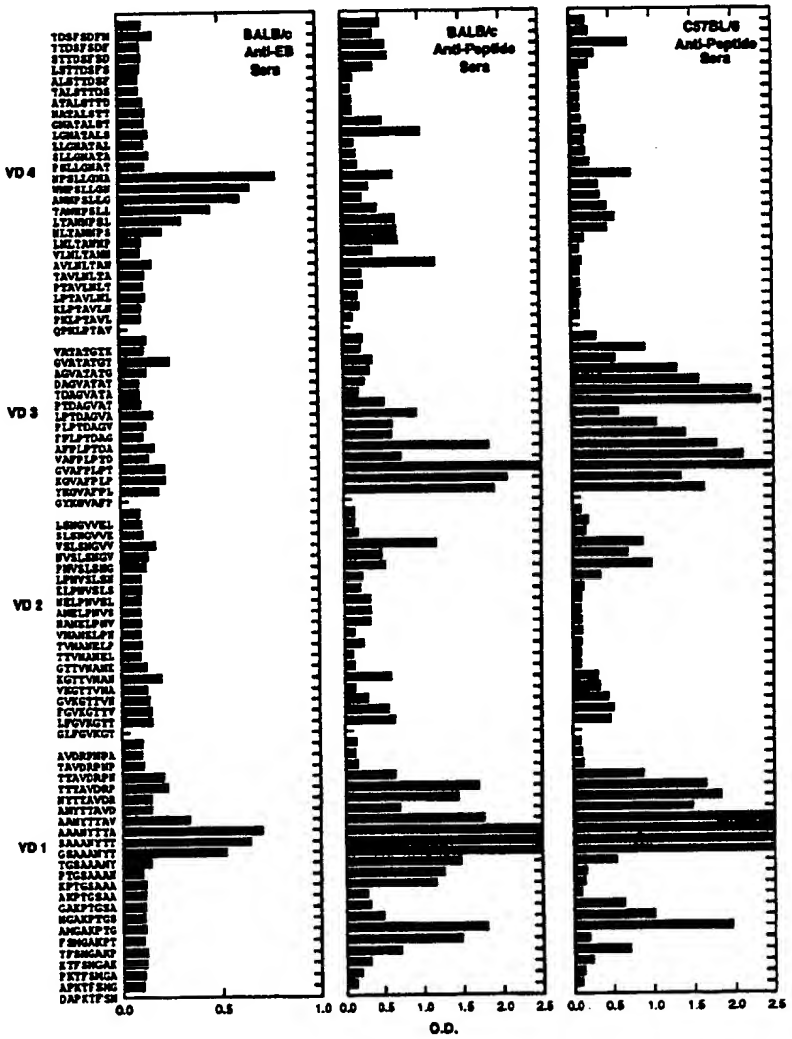

FIG. 2. Pepscan of octameric peptides representing the VDs of the MOMP of *C. pneumoniae*. The left panel represents the reactivity of pooled antisera from BALB/c mice to EBs of *C. pneumoniae* obtained on day 42 of the immunization protocol. The middle and right panels represent the pepscans generated with pooled antisera to the KLH-conjugated VD peptides raised in BALB/c and C57BL/6 mice, respectively, obtained on day 42 postimmunization. The sequence is from the N terminus at the bottom to the C terminus. O.D., optical density.

2). Interestingly, this was also the epitope recognized by antisera raised in BALB/c mice to viable EBs of *C. pneumoniae*.

VD2. The VD2 peptides were immunogenic whether used alone or conjugated to KLH. Both the peptide and KLH-peptide antisera were equally reactive to the MOMP in a Western blot. It is interesting to note that while the VD2 peptide antisera had higher ELISA titers compared with the VD1 peptide antisera, the Western blot to MOMP was weaker for the high-titered VD2 antisera, suggesting that much of the reactivity of the antibody raised to the VD2 peptide was unique to the peptide and not shared by MOMP. Antiserum from either mouse strain was not able to recognize EBs by ELISA or inclusion IFA, except for the KLH-VD2 antisera from C57BL/6 mice, which gave a very weak ELISA reaction. This lack of or very weak recognition of EBs could not be explained by antiserum that was low in titer since the titers were similar to those obtained with KLH-VD1. Therefore, it appears that the linear sequences recognized with the VD2 antisera are not available for binding on the surface of EBs. This finding is supported by the pepscan analysis of the anti-*C. pneumoniae* EB antisera, for which overall there was only minimal reactivity to the overlapping VD2 peptides (Fig. 2). The peptide antisera exhibited a strong peak of activity to the peptide sequence LPNVSLSNGV (data not shown). The KLH-peptide antisera also reacted to this region as well as the amino terminus of the peptide (Fig. 2).

VD3. When antiserum to the VD3 peptide was assayed by ELISA using the VD3 peptide and by Western blot to MOMP, the peptide alone was equally immunogenic in both strains of mice, as were KLH-conjugated forms. However, when assayed by ELISA using EBs, only the antiserum raised in C57BL/6 mice to KLH-VD3 was able to recognize the intact organisms, while by the less sensitive inclusion IFA this antiserum was negative. By pepscan, antisera from both strains of mice reacted with octapeptides within the VD3. Both strains reacted to the amino terminus of the VD3 region; however, only

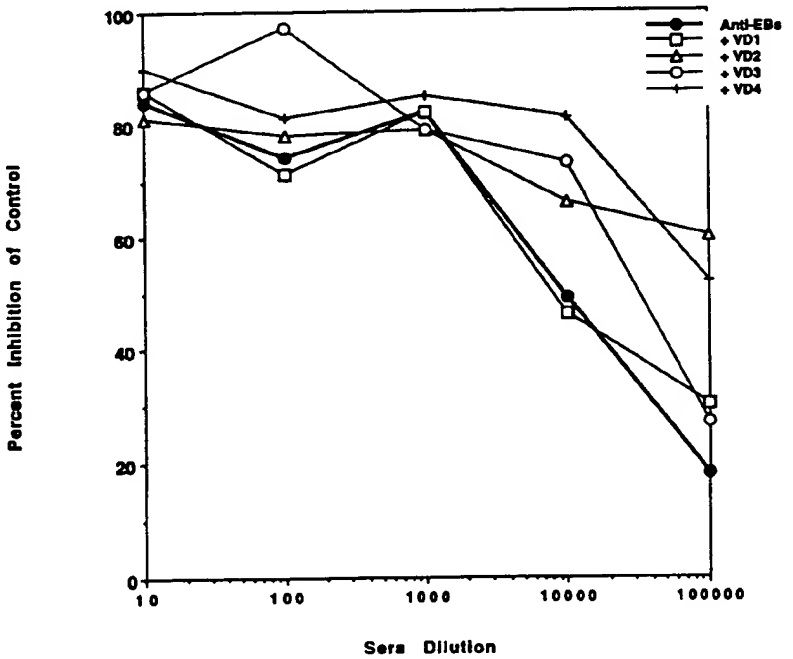

FIG. 3. Competitive in vitro neutralization assay obtained with BALB/c antisera to the *C. pneumoniae* EBs (TW-183). The antiserum to the EBs was tested in the presence or absence of the indicated peptides. The control antiserum was raised to KLH in Freund's adjuvant. Serum was obtained on day 42 of the immunization protocol. A reduction of >50% was considered positive for neutralization.

C57BL/6 mice reacted with the epitope(s) contained in the sequence DAGVAT (Fig. 2). Since the strengths of reactivity of these antisera from the two strains of mice were similar for the amino terminus of the peptide sequence, this would suggest that the epitopes in the sequence DAGVAT are most likely to be the surface-exposed area of VD3 since this was the region recognized only by the C57BL/6 antisera. The antisera raised to EBs in BALB/c mice had only minimal recognition of the VD3 overlapping peptides (Fig. 2), suggesting that in the native EBs this region appears to be weakly immunogenic compared with the VD1 and VD4 or that the native epitope(s) is highly conformational in nature.

VD4. Antisera to the unconjugated and KLH-conjugated VD4 peptides from both strains of mice reacted with the VD4 peptide, MOMP, and EBs, suggesting surface exposure of this VD. The pepscan of the antisera raised to EBs of *C. pneumoniae* showed a single area of reactivity to the region AWN PSLLGNA. The KLH-VD4 antisera likewise showed reactivity to this region; however, the reactivity, in general, was weak (Fig. 2). This region of the VD4 corresponds to the sequence TLNPTIA present in VD4 of *C. trachomatis*, recognized by neutralizing antibodies for members of the subspecies B complex. Therefore, it appears that VD4 is also surfaced exposed on *C. pneumoniae*.

In vitro neutralization. Antisera raised in BALB/c mice and C57BL/6 mice to the peptides and the KLH-conjugated peptides, as well as antisera to EBs of *C. pneumoniae* obtained from BALB/c mice, were used in an in vitro neutralization assay utilizing both HeLa and HEp-2 cells. While antiserum to EBs was able to neutralize *C. pneumoniae* up to a 1/10,000 dilution, there was no neutralization with any of the peptide antisera tested. When peptides representing the VDs were used in a competitive inhibition neutralization, as can be seen in Fig. 3, the VD peptides were not able to reduce the neutralizing ability of the *C. pneumoniae* EB antisera, providing further evidence that the linear peptides corresponding to these regions do not represent the native neutralizable epitopes.

DISCUSSION

Since its initial isolation, *C. pneumoniae* has become recognized as a leading cause of pneumonia, responsible for up to 10% of community-acquired pneumonias. Seroepidemiological studies have shown that 50% of adults have had exposure to this human pathogen (13). The most common age for acquisition of this organism is school age. This organism has also been associated with adult reactive airway disease, pharyngitis, bronchitis, and sinusitis (13). Animal models using intranasal inoculation of this pathogen have shown systemic spread with recovery of organisms from the spleen and peritoneal macrophages (25). Recently, there has been evidence presented that this organism can be found in atheromatous plaques, and thus the question of its association with coronary artery disease has been raised (12). Therefore, considering its importance as a human pathogen, studies on the basic structure and function of key components of this organism are necessary if we are to understand the interaction of this pathogen with the human host and if prevention strategies in the form of a vaccine are to be developed.

Antibodies that neutralize the infectivity of *C. pneumoniae* in vitro have been described. Puolakkainen et al. (20) identified two MAbs, RR-402 and TT-205, that were species specific and were able to neutralize *C. pneumoniae*. These authors, however, were unable to identify the antigen to which the MAbs were directed. They concluded that the reactive determinants were destroyed during attempts to characterize them and that the epitopes to which these antibodies are directed

are most likely conformational in nature. Unlike with *C. trachomatis*, no MAbs recognizing the MOMP of *C. pneumoniae* and able to neutralize this organism have been described.

The MOMP of *C. trachomatis* and *C. pneumoniae* is the predominant protein in the outer membrane and thus has the potential of being an important component of host-bacterium interactions (1, 15). DNA sequence analysis of the MOMP of *C. trachomatis* has revealed constant domains interspersed with four VDs (24). On the basis of sequences of the VDs the strains of *C. trachomatis* can be placed into four main groups (26). The VDs have been shown to contain epitopes that bind to MAbs that can neutralize the infectivity of the organism by in vitro or in vivo assays. Thus, in this species the MOMP has been the focus of several studies relating to its potential as a vaccine candidate (17, 27).

The MOMP DNA of *C. pneumoniae* has been shown to be conserved with as little as 5% variation among strains. In one study by Gaydos et al. (5) a 205-bp region that spanned VD4 was sequenced from 13 strains. These authors found VD4 sequences to be identical in the strains examined. Sayada et al. (22) examined the MOMPs of 11 *C. pneumoniae* strains of diverse geographical origins and found no difference in the restriction profiles of the PCR products generated. While this lack of variation within the MOMP of *C. pneumoniae* would be advantageous in attempts to define stable vaccine candidates that would lend protection to all strains of the organism, it also suggests that there is not much immunological pressure on the MOMP and therefore this protein may be less important in the host response (4).

It appears that there is variation in the host response to the MOMP of *C. pneumoniae*. Campbell et al. (2) reported the MOMP not to be an immunodominant protein in humans in terms of antibody response. Iijima et al. (9), however, found that some but not all patients responded with a strong antibody response to the MOMP of *C. pneumoniae*. In our studies, in which mice were immunized with EBs of *C. pneumoniae*, by Western blot the MOMP was immunodominant. These differences may just reflect a difference in the host immune response or may be a function of the route and dose used. It is also interesting to note that the two mouse strains used in this study, which differed in H-2 genotype, produced very different responses to the VD3. While both strains produced antibody to VD3, the molecular specificities of these antisera as seen by pepscan differed, and only that from the C57BL/6 mice recognized EBs. Therefore, some differences in host response may be in part due to the genetic background of the host, possibly (as in the mouse) related to HLA type.

The data we obtained with the pepscan with high-titered BALB/c mouse antisera to *C. pneumoniae* EBs suggest that epitopes in VD1 and VD4 were immunodominant and therefore possibly surface exposed. In examining the antisera to the peptides representing the VDs, it was also logical to conclude that VD1, VD3, and VD4 are surface exposed since antisera raised to peptides representing them were able to bind to EBs. The epitope in VD3 that is surface exposed was immunogenic in C57BL/6 mice but not in BALB/c mice. Therefore, these VDs have the potential to interact with the host cell and thus are a logical area to study in terms of neutralization.

Linear peptides representing surface-exposed VDs of *C. trachomatis* have been shown to elicit antiserum that was able to neutralize the infectivity of this organism in vitro (17, 21, 27). In addition, these peptides have been able to inhibit to some extent the neutralizing ability of polyclonal sera to EBs as well as neutralizing MAbs to VD1 of *C. trachomatis* serovar C and VD4 of serovar E (17, 21). The fact that peptides representing the linear binding site of the MAbs were not able to compete completely for the neutralizing ability of the MAbs suggests that there is a conformational component of the native neutralizable epitope. The data we presented with the peptides corresponding to the VDs of *C. pneumoniae* showed that antisera to these peptides were not able to neutralize the infectivity of this organism. In addition the linear peptides were not able to compete with EBs for the neutralizing antibodies present in polyclonal sera raised to EBs. Possible explanations for these findings are that either the VDs of the *C. pneumoniae* MOMP do not elicit neutralizing antibodies or that, if they do, the epitopes recognized are conformational in nature and thus not represented by short linear peptides. Here it is interesting to point out that the immunodominant region within VD4, as seen in BALB/c antisera to EBs, is the analogous region of the serovar E VD4 that elicits neutralizing antibodies (17). It is also important to note that this epitope was immunodominant, as seen by pepscan in antisera raised to the EBs of *C. pneumoniae*, but the response to the linear peptide was not dominant for this epitope, suggesting the conformational nature of this epitope in *C. pneumoniae*.

Therefore, it appears that, unlike with *C. trachomatis*, short peptides representing VDs of the MOMP of *C. pneumoniae*, as used in this study, without consideration of the possible conformational components, are not able to stimulate a humoral neutralizing response. Whether they are able to stimulate a protective cellular immune response will need to be investigated. However, because of their abundance in the outer membrane and surface exposure, as shown by this work, the role of the VDs in the interaction with the host needs further investigation.

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